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Genetic divergence and aggressiveness within a supercolony of the invasive ant *Linepithema humile*

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Abstract

Biological invasions constitute an opportunity to study the evolutionary processes behind species' adaptations. The invasive potential of some species, like the Argentine ant (*Linepithema humile*), has likely been increasing because they show low intraspecific competition. However, multiple introductions over time or genetic divergence could increase the probability of intraspecific competition, constituting barriers for their dispersal and thus, decreasing invasive success. Here, we studied the genetic and behavioural variability of *L. humile* workers collected at six locations on the NW coast of the Iberian Peninsula, a possible scenario for multiple introductions and population divergence, due to its high level of maritime traffic and complex coastal geography. We analysed behaviours related to spatial navigation (exploration, wall-following), resources acquisition, and competition (inter and intraspecific aggressiveness) through two relevant seasons for the nest ecology: spring and autumn. Genetic analyses using microsatellites indicated that the nests studied belonged to the most spread supercolony in South Europe. However, we identified the existence of two genetically differentiated clusters in Galiza. Lethal interactions were found between workers from different and similar genetic clusters, but a trend suggests higher agonistic behaviours between the two genetic groups. Genetic differences were positively correlated with the geographical distance, but aggressiveness was not correlated with any of them. Ants from each of the tested nests expressed different behaviours with high plasticity through time. Ants from all nests showed more exploration and aggressiveness, less wall-following and faster detection of food in autumn than in spring, with no intraspecific aggressiveness observed in spring. Our findings suggest competition between nests of the same supercolony

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and behavioural seasonal variability, supporting the hypothesis of divergent evolutionary processes. The results of our work question the assumed unity of supercolonies of this species and offer insights for understanding the future adaptation of *L. humile* in the introduced areas.

Keywords
intraspecific competition, population genetics, subcolony, unicoloniality, variability

Introduction

The spread of exotic invasive species constitutes one of the most serious threats to biodiversity (Dueñas et al. 2021), bringing with it high economic impacts (Bradshaw et al. 2021; Haubrock et al. 2021; Angulo et al. 2022). The introduction of exotic invasive species has greatly increased in the last decades due to market globalization and its synergy with climatic change (Galil et al. 2007; Perrings et al. 2010; Ramsfield et al. 2016). For invertebrates, traits like their small body size, large population numbers, or high reproductive potential hinder the application of control programs (Hoffmann et al. 2016) and therefore, many exotic invertebrates become pests in their introduced areas. In particular, social insects are among the organisms with the highest representation and the greatest threat to ecosystems (Siddiqui et al. 2021). Invasive ants displace native species, alter ecosystem services (food web, recycling, pollination), protect species considered as pests (e.g. aphids), invade human installations, and transmit pathogens to other species, causing ecological costs valued at US$ 10.95 billion over the last 90 years (reviewed in Pedersen et al. 2006; Baty et al. 2020; Angulo et al. 2022).

One of the most worrying points in conservation programmes, albeit one that is extremely interesting for science, is the evolution of the introduced species (e.g. Eurohornet project; Wystrach and Lihoreau 2020). Invasive species succeed in adapting to newly colonized environments in a very narrow window of time, especially in human dominated ecosystems (Pyšek et al. 2010), usually developing higher fitness than native species (Boltovskoy et al. 2020). The evolution of introduced-invasive ants allows us to record complex evolutionary processes at a human time scale as local adaptation and intraspecific divergence (Helanterä 2022), and to understand the species trade-offs, which could constitute a key factor in the fight against the ecological and economic problems that these species cause.

One of the most relevant examples of invasion due to social organization emerged between 1882 and 1891 with the introduction of the Argentine ant (*Linepithema humile* Mayr, 1868) into Madeira and New Orleans (Newell and Barber 1913; Suarez et al. 2001). This first introduction event was followed by others which quickly spread this species around the world (Suarez et al. 2001). The success of *L. humile* is related to a change in its intraspecific competition. In its native distribution, *L. humile* colonies are composed of connected nests extending up to 500 m ca. (unicolonial supercolonies; Pedersen et al. 2006). Although there is no evidence of individual or resources flow between long distanced nests (Moffett 2012), individuals do not compete or attack

when belonging to the same supercolony while they show high aggressiveness towards members of other supercolonies (Pedersen et al. 2006). However, only a small number of vast supercolonies extend over thousands of kilometres in the introduced areas (e.g. Giraud et al. 2002; Thomas et al. 2007; Blight et al. 2010). The way in which this new ecological scenario has emerged is still under debate, although it is assumed to be the product of a colony founding and genetic bottleneck (Suarez et al. 1999) possibly followed by the dominance of colonies with reduced genetic diversity (Tsutsui et al. 2003) or selection pressures under high densities (Giraud et al. 2002; but see Thomas et al. 2006). The invasive potential of these vast supercolonial organisations (Holway et al. 1998) resides in their capability for unrestricted growth (Moffett 2012), thus making *L. humile* one of the most dangerous introduced species (Lowe et al. 2000). Even if it is possible to reduce its presence in the introduced areas (Tatsuki et al. 2012; Hoffmann et al. 2016; Angulo et al. 2019), most authors agree with the impossibility of its eradication and therefore, only preventive methods to fight against the expansion of this species can be proposed (Siddiqui et al. 2021).

Although it was proposed that the introduction of new supercolonies would not interfere with the expansion of well-established supercolonies (Moffett 2012), the existence of several supercolonies in the same geographic area leads to resource limitation and the death of millions of individuals per year at the border between supercolonies (Thomas et al. 2007). Therefore, the emergence of new supercolonies may induce intraspecific competition – the missing characteristic of the introduced supercolonies – and reduce the species environmental effects (Moffett 2012; Helanterä 2022). The success of the invasive potential of *L. humile* could be (theoretically) disrupted by two main processes: (i) multiple introductions of native colonies that maintain competition in the introduced area (e.g. Buczkowski et al. 2004; Vogel et al. 2009) and (ii) the divergent evolution of colonies already established in the introduced areas caused by local adaptations (see Ingram 2002; Moffett 2012; Helanterä 2022) and the lack of genetic flow between colonies (see Heller 2004; Pedersen et al. 2006). We did not consider the foundation of new supercolonies by flying queens of the established supercolonies due to the unexpectedness of this process (Markin 1970; Helanterä 2022). In the first case, assuming *L. humile* is introduced via maritime flow, a high number of competitive colonies would be expected in coastal regions with commercial harbours and high genetic diversity might be expected in these areas (see Moffett 2012), as happens on the Californian coast (Suarez et al. 2002; Thomas et al. 2007). In the second case, however, lower competition between colonies would be expected due to the dependence of divergent evolution on long-term isolation processes. This process was suggested as the cause of emergence of new supercolonies in Europe (Moffett 2012) due to the similar genetic diversity, chemical cuticular composition, and low aggressiveness occurred between the Corsican supercolony and the main supercolony (Blight et al. 2010). In both cases, variability in behaviour between colonies may be expected, with higher variability in the case of adaptation of native colonies to newly colonized environments (multiple introductions) than in the case of local environmental adaptations of previously established colonies (evolutionary divergence).

Linepithema humile shows behavioural variability both in its native and introduced areas (Blight et al. 2017) and in the latter, also among (Giraud et al. 2002; Blight et al. 2010) and within supercolonies (Chen and Nonacs 2006; Thomas et al. 2007; Van Wilgenburg et al. 2010a). As workers' phenotypic plasticity conditions the colony behaviour (Pinter-Wollman 2012), local environmental differences may lead to local adaptations and thus, to divergence and competition (see Helanterä 2022). Behavioural divergence due to differences in spatial location might increase variation in colony productivity or food consumption, both factors that increase variation in cuticular compounds (Liang and Silverman 2000; but see Giraud et al. 2002; Buczkowski et al. 2005; Walsh J et al. 2020) and therefore, act as modifiers of the interactions between individuals from distanced nests of the same supercolony.

Seasonality constitutes a further environmental factor able to modify ant colony behaviour (Markin 1970; Benois et al. 1973; Thomas et al. 2005, 2006, 2007; Heller and Gordon 2006) and individual cuticular compounds (Abril et al. 2018), possibly leading to changes in the ants' invasive potential (Thomas et al. 2006). Understanding how locally adapted nests deal with seasonal changes would help to decipher present divergent responses but also the future scenario modified by the effect of climatic change. In a similar way, environmental changes due to climatic trends could modify the costs and benefits for invasive species and open new distribution areas (Thomas et al. 2006).

In this study, we focus on ethological and genetic analyses of *L. humile* workers collected at six different localities in Galiza (NW Iberian Peninsula), an area where the biology of the species is poorly known (Giraud et al. 2002; Gómez and Espadaler 2004). Galiza constitutes a relevant point of maritime commercial flow between South America and Europe, increasing the likelihood of the introduction of exotic species and the establishment of colonies of multiple origin (Castro et al. 2017). If multiple introductions of *L. humile* from its native range have occurred in Galiza, we would expect to find a high number of well-limited genetic and behavioural groups. Galiza's coastal configuration is characterized by estuaries and habitat diversity, which could favour allopatric and sympatric events respectively. If the nests of the main supercolony (the supercolony present in this region; see Giraud et al. 2002) suffered local adaptations and allopatry, we would expect a progressive genetic and behavioural diversity. To test these hypotheses, we carried out a set of behavioural tests in six sampled nests of *L. humile* from Galiza, similarly to previously published works (Blight et al. 2017). We controlled for seasonal effects by studying the behaviour of workers from the studied nests after and before winter. To verify if the lack of aggressiveness between conspecifics of the different nests studied was due to general loss of aggressiveness (for instance, linked to seasonal effects; Ichinose 1991), we analysed the aggressiveness of *L. humile* against a native species (*Myrmica rubra* Linnaeus, 1758). Finally, to analyse the genetic variability and population structure within our study area, we genotyped individuals from the nests sampled in Galiza, together with samples belonging to the two supercolonies previously identified in the Iberian Peninsula (Giraud et al. 2002) at seven microsatellite loci.

Materials and methods

Specimen collection

Individuals from one ant nest were collected in March and September 2020 at each of four locations (Carril Garden, Ribeira, Pontevedra and Reboreda), distanced by approximately 30 km on a latitudinal N-S axis following the Galizan Southern coast (see Fig. 1; Table 1). In a preliminary test performed in the laboratory in August 2019 with marked ants, workers belonging to Carril Garden killed 100% of workers from Pontevedra (N = 20). Given these results, we aimed to determine if the high aggressiveness showed by individuals from Carril Garden could be also expressed in other nests geographically close; or if this was a characteristic of this particular nest. To this aim, we collected individuals from two nests located at approximately 1–2 km from Carril Garden (Carril Coast and Trabanca). We assumed each nest was maintained in the same place across seasons (see Vogel et al. 2009), but we did not analyse the flow of individuals between nests. Ants were collected using small shovels and brushes.

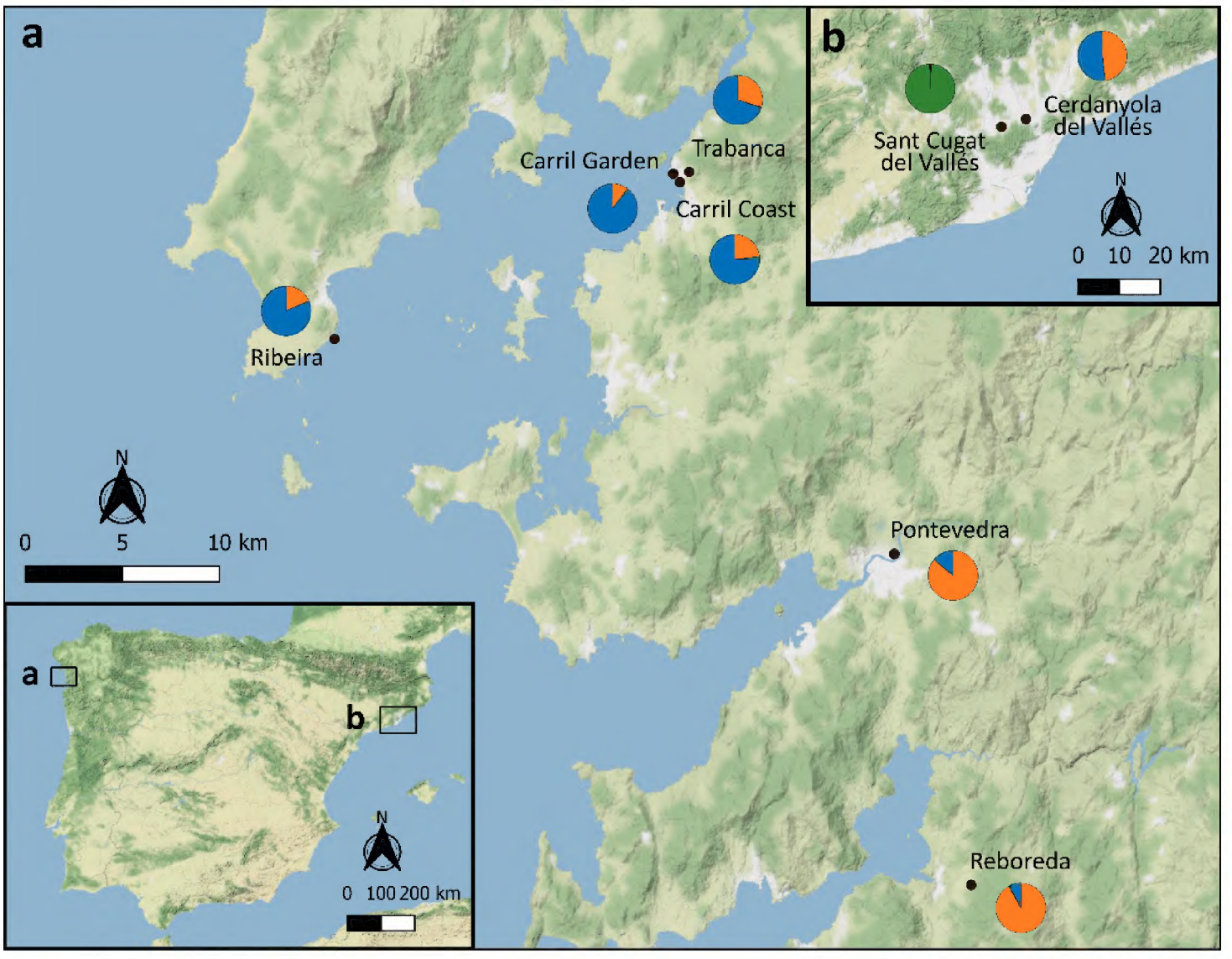

Figure 1. Map showing the location (black dots) of the eight colonies of *Linepithema humile* sampled for this study and listed in Table 1. Pie charts by each locality illustrate the proportion of individuals from each locality that were assigned to each of the genetic clusters identified by STRUCTURE (see Results and Fig. 7 and Suppl. material 1: Fig. S3). The figure has been created using QGIS version 3.22.3 (Anon 2022).

Table 1. Details on the *Linepithema humile* colonies sampled for this study shown in Fig. 1. Listed are the locality name, the type of environment and the coordinates for each sampled population. The order in which the colonies sampled in Galiza are listed follows a N-S latitudinal gradient (see Fig. 1). For the two localities sampled in Catalunya, the acronym in brackets refers to whether they belong to the Catalonian (CS) or the main supercolony (MS).

Region	Locality	Environment	Coordinates
Catalunya	Sant Cugat del Vallés (CS)	garden	41°28'29"N, 2°04'39"E
	Cerdanyola del Vallés (MS)	garden	41°29'29"N, 2°08'54"E
Galiza	Trabanca	agriculture	42°36'56"N, 8°45'55"W
	Carril Garden	garden	42°36'52"N, 8°46'29"W
	Carril Coast	coast	42°36'39"N, 8°46'16"W
	Ribeira	coast	42°32'19"N, 8°59'12"W
	Pontevedra	garden	42°26'23"N, 8°38'14"W
	Reboreda	garden	42°17'14"N, 8°35'21"W

Fluon-coated open plastic boxes (18 × 18 × 8 cm) were used to transport the ants to the laboratory. Ants were maintained in the same plastic boxes used for collection, with soil from their own nests (following Giraud et al. 2002) and tubes filled with water and cotton covered with paper as artificial nests. Food (based on Bhatkar and Whitcomb 1970) was provided *ad libitum*. Nests were kept in the laboratory at room temperature, with a photoperiod of 12:12 h. Additionally, samples belonging to the two *L. humile* supercolonies identified in the Iberian Peninsula (Giraud et al. 2002) were collected on 10 March 2021 in Catalonia (Cerdanyola del Vallés and Sant Cugat del Vallés; see Table 1), to be used for the genetic analyses.

Exploration and thigmotaxis tests

In each season, spring (March) and autumn (September), fifty workers from each nest were randomly collected from the outside and inside of the tubes from the experimental nests to avoid biased selection of workers (foragers and nurses). Experimental workers were individually placed in Fluon-coated Petri dishes (Ø = 5.5 cm). Ten minutes of free walk observation were recorded with a Canon Legria HF M56 video camera. Tests were performed daily at 10:00 a.m. for six days. From the videos, we extracted the position of the individuals at a frame rate of 2.08 frames per second using the software Swistrack version 4.0 (Correll et al. 2006). Data were then analysed with R version 4.0.2 (R Core Team 2021) to measure the proportion of time individuals spent moving as a proxy of exploration (locomotor activity under non-risky and novel environments; Réale et al. 2007). An individual was considered as moving if it moved more than 1 mm between two frames. We selected the frequency of movements, rather than the total distance covered by each individual, to avoid effects related with ant body size. We also calculated the time spent far from the dish border (5 mm) as a proxy of border avoidance (i.e. the opposite to wall-following; also see Sanmartín-Villar and Jeanson 2021). Ants navigate their environment by contacting structures (Dussutour et al. 2005), so abandoning the spatial reference might be linked with cognitive performance (Doria

et al. 2019) or personality traits as boldness (Valle 1970; Walsh and Cummins 1976; Sneddon 2003; Carlson and Langkilde 2013; Detrain et al. 2019), factors that might affect individuals' dispersion.

Foraging efficiency test

Two hundred workers from each nest were randomly collected (see above) and placed in groups of 10 in 20 Fluon-coated Petri dishes (Ø = 13 cm) connected with another Fluon-coated Petri dish (Ø = 5.5 cm) by a plastic bridge (5.54 × 2.51 × 1.1 cm). A tube containing water and covered with paper was added to the bigger dish. The focal ants were kept for one day in this experimental setup to get familiarised with the new arenas and to experience a similar period of starvation to standardise their food requirements and thus, their feeding drive. After 24 h, we added food to the small dish. We video recorded the first 30 minutes after adding the food and took pictures of the small dishes 10 min after we stopped recording. We analysed the time needed for the first worker to reach the food and how many individuals were inside the dish containing food after 40 min as a proxy for foraging efficiency (hereafter, "number of foragers"). Tests were performed in March and September at 10:00 a.m. over three consecutive days, testing workers of two nests per day.

Aggressiveness test

To test the aggressiveness between different nests, 100 individuals were randomly collected from each nest and paired with an individual from another nest (N = 20 for each possible pair's combination). This procedure was performed in both seasons. Pairs were placed in Fluon-coated Petri dishes (Ø = 5.5 cm). The time that elapsed between the introduction of the first and second individual was approximately one second. To avoid the residency effect (Shreeve 1987; Peixoto and Benson 2012; but see Kemp and Wiklund 2004), we alternated the order of each individual's addition. Control pairs consisted of two workers from the same nest (N = 10 replicates in March, N = 20 in September). To analyse the seasonal effect, workers from the same nest but collected at different times (March and September) were paired in September (N = 220; 20 per season and nest except for Carril Garden nest, in which no individuals collected in March survived). We used the same procedure to confront workers from each nest with *Myrmica rubra* workers. Interactions within individual pairs were analysed by video recording the first 10 min after the individuals' addition. The video allowed us to identify individuals' nest origin without marking them by following them until the first attack or until the end of the video. We recorded the presence or absence of bites between paired ants on each dish as an indicator of aggressiveness. We compared aggressiveness between nests by comparing the number of dishes where we had observed attacks. We did not record the time engaged on each attack (see Hakala et al. 2020) or posterior attacks produced in the same dish - only the first attack was recorded on each pair - to avoid considering behaviours conditioned by the first attack or repeated measures

(experiences). We did not study other interactions (antennation, touch, trophallaxis, avoidance, gaster elevation) because previous works that considered these ended up merging their score values (Carlin and Hölldobler 1986; Holway et al. 1998; Giraud et al. 2002; Roulston et al. 2003; Vogel et al. 2009; Blight et al. 2012, 2017; Hakala et al. 2020) or interpreted them as binomial interactions (i.e. aggressive or non-aggressive; see Suarez et al. 1999, 2002; Tsutsui et al. 2000, 2003; Thomas et al. 2005, 2006, 2007; Wetterer and Wetterer 2006; Blight et al. 2010). We considered that only measuring attacks is a conservative method that, even if it underestimates the behavioural response during interactions, reflects the agonism of the encounter and alleviates artefacts (for instance, stress produced by handling; Bernadou et al. 2018) and subjective interpretations. Paired individuals were maintained for 24h with wet cotton to check mortality (Blight et al. 2010).

Observers were distanced from the ants when performing all behavioural tests described above and wore gloves and masks when manipulating the individuals to avoid impregnating body waxes and exhaling in their direction (Chen and Nonacs 2006). We assumed ant manipulation did not elicit aggressiveness (Heller 2004; Bernadou et al. 2018) or if it occurred, we assumed the same effect for all workers tested.

DNA extraction, PCR, and microsatellite genotyping

Total genomic DNA was extracted from 24 workers from each nest using the GeneJet DNA extraction kit (ThermoFisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. Genetic variability was assessed by using seven microsatellite polymorphic loci: *Lihu-S3*, *Lhum-11*, *Lihu-T1*, *Lhum-13*, *Lhum-19*, *Lihu-M1* and *Lhum-62* (Krieger and Keller 1999; Tsutsui et al. 2000). PCRs were carried out in a total 10 µl volume containing 5 µl of DreamTaq Green PCR Master Mix (ThermoFisher Scientific), 0.2 pmol of each primer, and ~10 ng of genomic DNA. Cycling conditions consisted of 5 min at 94 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at T_a °C and 30 s at 72 °C, and a final extension of 2 min at 72 °C (where T_a is the marker-specific annealing temperature; see Suppl. material 1: Table S1). PCR products were pooled into one of two genotyping panels, depending upon the expected allele sizes and the 5' fluorescent dye (6-FAM, NED, PET or VIC; Applied Biosystems, Foster City, CA, USA); along with GeneScan-500 LIZ size standard (Applied Biosystems). Products were separated using capillary electrophoresis on an ABI3130 automated DNA sequencer (Applied Biosystems) at the CACTI genomics facility from the University of Vigo. Allele bins and sizes for each locus were determined with the 3rd order least squares method implemented in the microsatellite plugin from Geneious version 9.1.8 (Kearse et al. 2012).

Genetic diversity and population structure analyses

Observed and expected heterozygosities, the number of private alleles for each locus and deviations from Hardy-Weinberg equilibrium (HWE) for each locus in each

location were calculated using Arlequin version 3.5 (Excoffier et al. 2005). The Bayesian model-based clustering approach implemented by Structure version 2.3.4 (Pritchard et al. 2000) was used to identify genetic clusters while assigning individuals to each of these clusters. Structure analyses were carried out including all the genotyped localities (i.e., Catalonian and main supercolony samples plus the nests sampled in Galiza), in order to determine whether the samples of Galiza belonged to the main or the Catalonian supercolony. We used the admixture model, and the number of clusters (K) was estimated by comparing the log-likelihood ratios in two independent runs for values of K between 1 (panmixia) and 8 (the total number of sites sampled). Each run consisted of 5^6 iterations, with a burn-in period of 5^5 iterations, to ensure convergence of the Monte Carlo Markov Chain (MCMC). A second run of Structure was carried out to analyse the genetic structure within samples from the main supercolony (i.e., excluding Sant Cugat del Vallés) as determined from the previous analysis; with four independent runs for values of K between 1 and 3. The scale of major population subdivision within our datasets (*i.e.* the value of K that maximizes the posterior probability of the data) was calculated following the method of Evanno et al. (2005), with ΔK calculated using Structure Harvester (Earl and vonHoldt 2012; available at http://taylor0.biology.ucla.edu/structureHarvester/).

Pairwise F_{ST} values were calculated in Arlequin version 3.5 (Excoffier et al. 2005), using the number of alleles distance method and with significance assessed by 1,000 permutations. Additionally, we calculated the level of genetic similarity between colonies as the percent of shared alleles (i.e., the number of alleles shared across loci between localities/total number of alleles possessed by both localities; as in Tsutsui et al. 2000).

Geographical distances

The geographical distances between the nests sampled in Galiza were estimated using QGIS version 3.22.3 (Anon 2022), either by measuring the shortest distance (beeline, considering ants displacement by the sea) or by measuring the length of the shortest terrestrial path connecting colonies. The two methods differ mainly in the consideration of the location of the Ribeira nest.

Statistical analyses

Statistical analyses were performed using R version 4.0.2 (R Core Team 2021). We ran linear models (LMs) using exploration (proportion of time spent moving) or lack of thigmotaxis (proportion of time spent out of the arena edge) as response variable, while nest and season were selected as fixed factors in the analyses. Nests were compared by pairs in *post hoc* analyses using the *emmeans* package (Lenth 2021). We compared the time needed to reach the food for the first ant of each replicated group in the foraging tests using survival curves with the Kaplan-Meier method (Kassambara et al. 2021). The total number of foragers present in the feeding dish after 40

min of food addition and aggressiveness (total number of ants performing the first attack) were analysed for each season by contingency chi-squared tests with Holm's correction. For the tests of aggressiveness, the relationship between the number of pairs with aggressions and the genetic similarity (percentage of shared alleles) or the geographic distances (both beeline and terrestrial) were assessed with a generalised linear model (GLM) with binomial data and logit link function. Proportions of pairs with aggressions was the response variable and the three different distances were fixed factors. Interactions between genetic and both geographic distances were considered but we removed them due to the lack of significance. We used F_{ST} values, which are the proportion of the total genetic variance contained in a subpopulation (S) relative to the total genetic variance (T) (Wright 1949), as a measure of the degree of genetic differentiation between nests. To have a general view of the nests behavioural differences between seasons, we carried out principal component analyses (PCAs) for all behaviours measured (exploration, thigmotaxis, first forager, number of foragers, and interpopulation and interspecific aggressiveness) by using the *prcomp* function (stats package).

Data availability

Data are provided as supplementary information. Information on genotypes for the sampled populations is available upon request to the authors.

Results

Exploration and thigmotaxis tests

Workers' movement frequency differed among the six studied nests ($F_{5;587} = 7.28$, $p < 0.001$; Table 2), between seasons ($F_{1;587} = 398.36$, $p < 0.001$), and there was an interaction Nest × Season ($F_{1;587} = 6.70$, $p < 0.001$, $R^2 = 0.44$; Fig. 2A). Workers from all nests increased their exploration in autumn (differences ranging between 19% up to 42%, $p < 0.001$).

The use of the border by workers differed among the six studied nests ($F_{5;592} = 2.64$, $p = 0.022$, $R^2 = 0.08$; Fig. 2B; Table 2). Workers used more the edge of the arena in spring ($F_{1;592} = 40.96$, $p < 0.001$), but no significant differences were found for the interaction Nest × Season ($F_{5;587} = 1.66$, $p = 0.142$).

Foraging efficiency test

Workers' first arrival at the food differed among nests (spring: $\chi^2 = 23.4$, df = 5, $p < 0.001$, Fig. 3A; autumn: $\chi^2 = 14.1$, df = 5, $p = 0.020$, Fig. 3B; Table 2). Workers reached the food faster in autumn than in spring (9.61% in average; $\chi^2 = 10.1$, df = 1, $p = 0.001$, Fig. 3C).

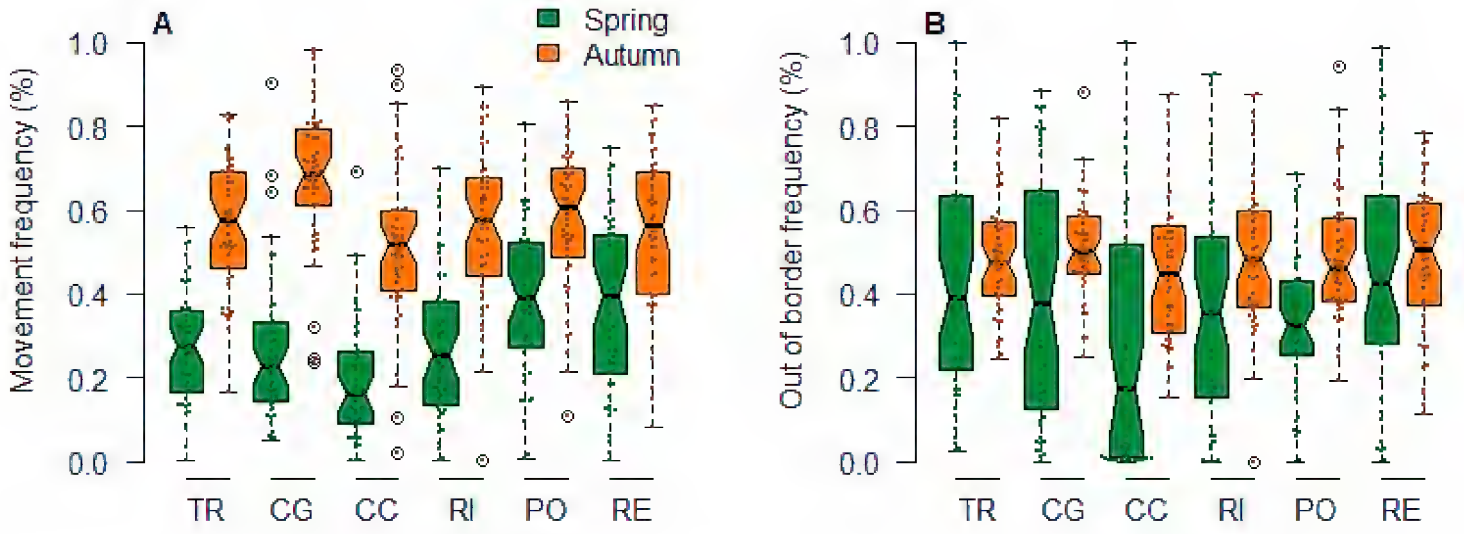

Figure 2. Frequency of movement (exploration **A**) and out of border frequency (proportion of time in which the ants did not use the arena border; thigmotaxis **B**) showed by each of the colonies of *Linepithema humile* from Galiza included in this study, in both spring and autumn seasons. The horizontal line in each box represents the median, and the lower and upper hinges indicate the first and third quartiles. Lower and higher whiskers extend to the most extreme values within 1.5 interquartile ranges from the first and third quartiles, respectively. Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

The number of workers reaching the food 40 minutes after its addition was different among nests (spring: $\chi^2_5 = 46.09$, $p < 0.001$; autumn: $\chi^2_5 = 41.73$, $p < 0.001$; Fig. 3D; Table 2). More workers (average 7.73%) were present in the feeding dish in spring than in autumn ($\chi^2_1 = 23.76$, $p < 0.001$).

Aggressiveness test

No fights were observed between paired *L. humile* workers during the 10 min of observations carried out in March. Mortality after 24 h was only found in dishes shared by workers from nests of Carril Coast and Pontevedra (7.5%) and from Trabanca and Pontevedra nests (10%).

In September, no fights were observed between paired workers of the same nest (control) during the 10 min of recorded observations. No fights were observed between individuals from the same nest but collected in different seasons (March and September) in Trabanca and Reboreda, while fights were observed in 10–20% of dishes when mixing individuals from different seasons in Carril Coast, Ribeira, and Pontevedra nests (Fig. 4A). Fights between workers collected in September were observed in all kinds of combinations confronting individuals from different nests, except the cases in which workers from Carril Coast nest were mixed with workers from Ribeira and Reboreda nests. The number of dishes in which attacks occurred depended on the nests pairs' combinations ($\chi^2 = 36.86$, df = 14, $p < 0.001$; Fig. 4A). Workers belonging to Carril Garden – Reboreda fought in 50% of pairs, followed by Carril Coast – Pontevedra (25%), Trabanca – Pontevedra and Carril Garden – Pontevedra (20%), Trabanca – Carril Garden and Carril Garden – Carril Coast (15%), Trabanca – Carril

Table 2. Between nests *post hoc* significant differences for all the behavioural variables measured, except aggressiveness. *P*-values correspond to Holm's corrected *p*-values. $N_{exploration} = 50$ (except for TR in spring, N = 49); $N_{out\ of\ border} = 50$; $N_{arrival\ to\ food} = 10$; $N_{number\ of\ foragers} = 10$. Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

Behaviour	Season	Nest pair				*p*-value
		Nest	Mean±SD	Nest	Mean±SD	
Exploration (%)	Spring	PO	38.67±17.93	TR	27.55±13.31	0.016
				CG	26.43±17.43	0.005
				CC	18.91±14.20	<0.001
				RI	27.93±18.02	0.020
		RE	37.16±20.54	TR	27.55±13.31	0.049
				CG	26.43±17.43	0.020
				CC	18.91±14.20	<0.001
	Autumn	CG	68.14±16.05	TR	56.76±15.63	0.015
				CC	52.30±18.91	<0.001
				RI	56.18±18.63	0.009
				PO	57.55±16.73	0.030
				RE	55.16±18.81	0.004
Out of border (%)	Spring	CG	39.49±29.23	RE	44.49±27.86	0.033
Arrival to food (s)	Spring	CG	13.12±10.56	TR	4.80±3.06	0.021
				RE	4.69±2.14	0.012
		RI	13.08±9.92	TR	4.80±3.06	0.001
				RE	4.69±2.14	0.001
Number of foragers	Spring	RE	3.65±1.57	CG	1.45±1.50	<0.001
				CC	1.75±1.55	<0.001
				RI	1.25±1.45	<0.001
		TR	2.55±1.57	RI	1.25±1.45	0.017
	Autumn	TR	2.75±1.83	CG	0.95±0.89	<0.001
				CC	1.45±1.47	0.024
				RI	0.80±0.77	<0.001
				PO	1.05±2.09	<0.001
				RE	1.35±0.99	0.001

Coast, Trabanca – Ribeira, and Pontevedra – Reboreda (10%), Trabanca – Reboreda, Carril Garden – Ribeira, Ribeira – Pontevedra, and Ribeira – Reboreda (5%). The number of attacks observed showed no significant correlation with the percentage of shared alleles or the geographical distances (terrestrial and the shorter distance) between nests ($p > 0.503$; see Fig. 5A, B). A similar proportion of attacks was observed within (9.29%) and between (16.2%) the North and South genetic clusters identified in Galiza (W = 33, p = 0.597). Workers from both clusters triggered the attack towards workers from the other clusters in a similar way (North = 60.87%, South = 39.13%; binomial test: $p = 0.405$).

Mortality after 24 hours differed among nests ($\chi^2 = 87.48$, df = 14, $p < 0.001$; Fig. 4B). The highest number of deaths was observed in pairs confronting Pontevedra with Carril Coast (35%) and Trabanca workers (30%), followed by Reboreda with Carril Garden (20%) and Carril Coast (17.5%). Fewer than 7.5% of workers died

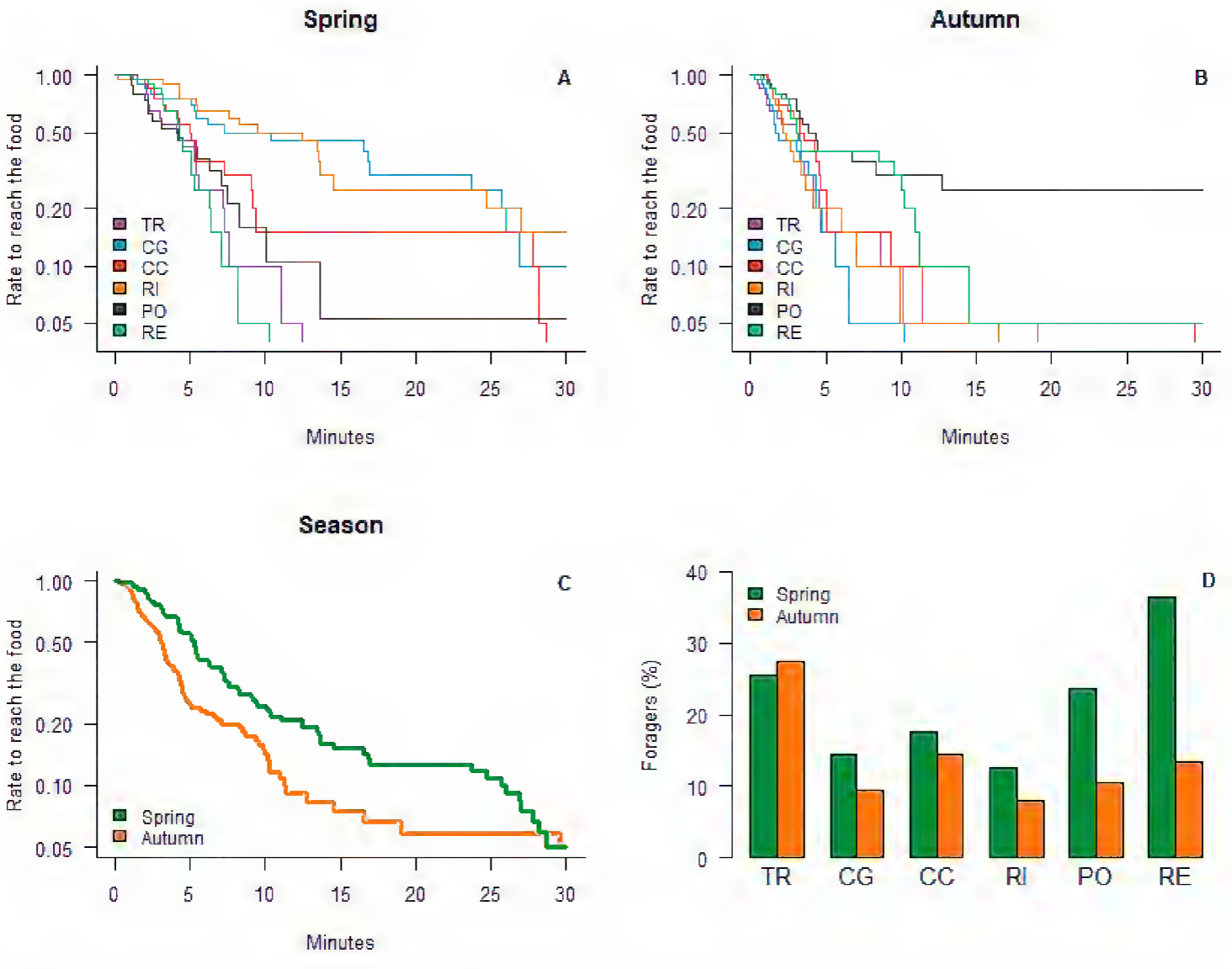

Figure 3. Rate of individuals reaching the food for the first time for each *Linepithema humile* nest sampled in Galiza in spring **A** and autumn **B** overall for each season **C** and proportion of workers from each nest present in contact with the food after 40 min of the food addition **D** Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

in the other combinations and no death events were recorded between Ribeira with Carril Garden or Pontevedra and between Reboreda with Trabanca or Pontevedra. A worker died in the Pontevedra control group in March while four died in September (Trabanca = 1, Carril Garden = 1, Ribeira = 2).

Linepithema humile engaged faster in fights with *Myrmica rubra* (0.73±1.21 min after being paired) than with their conspecifics from different nests (5.43±2.28 min; t = 12.79, df = 69.42, $p < 0.001$). *Linepithema humile* workers started more fights than *M. rubra* workers when they were mixed in spring (mean *L. humile* = 4.17±2.13, mean *M. rubra* = 1.00±0.63; $t_{5.87} = 3.48$, $p = 0.014$) and autumn (mean *L. humile* = 6.33±2.66, mean *M. rubra* = 2.33±1.75; $t_{8.56} = 3.08$, $p = 0.014$; Fig. 6). No differences between nests or seasons were found for the aggressiveness of *L. humile* towards *M. rubra* and *vice versa* ($\chi^2 < 8.16$, $p > 0.147$).

Linepithema humile workers died more than *M. rubra* workers when they were mixed in spring (mean *L. humile* = 9.17±3.6, mean *M. rubra* = 4.50±1.4; $t_{6.43} = 2.96$, $p = 0.023$) and autumn (mean *L. humile* = 14.00±1.9, mean *M. rubra* = 3.83±1.8; $t_{9.99}$

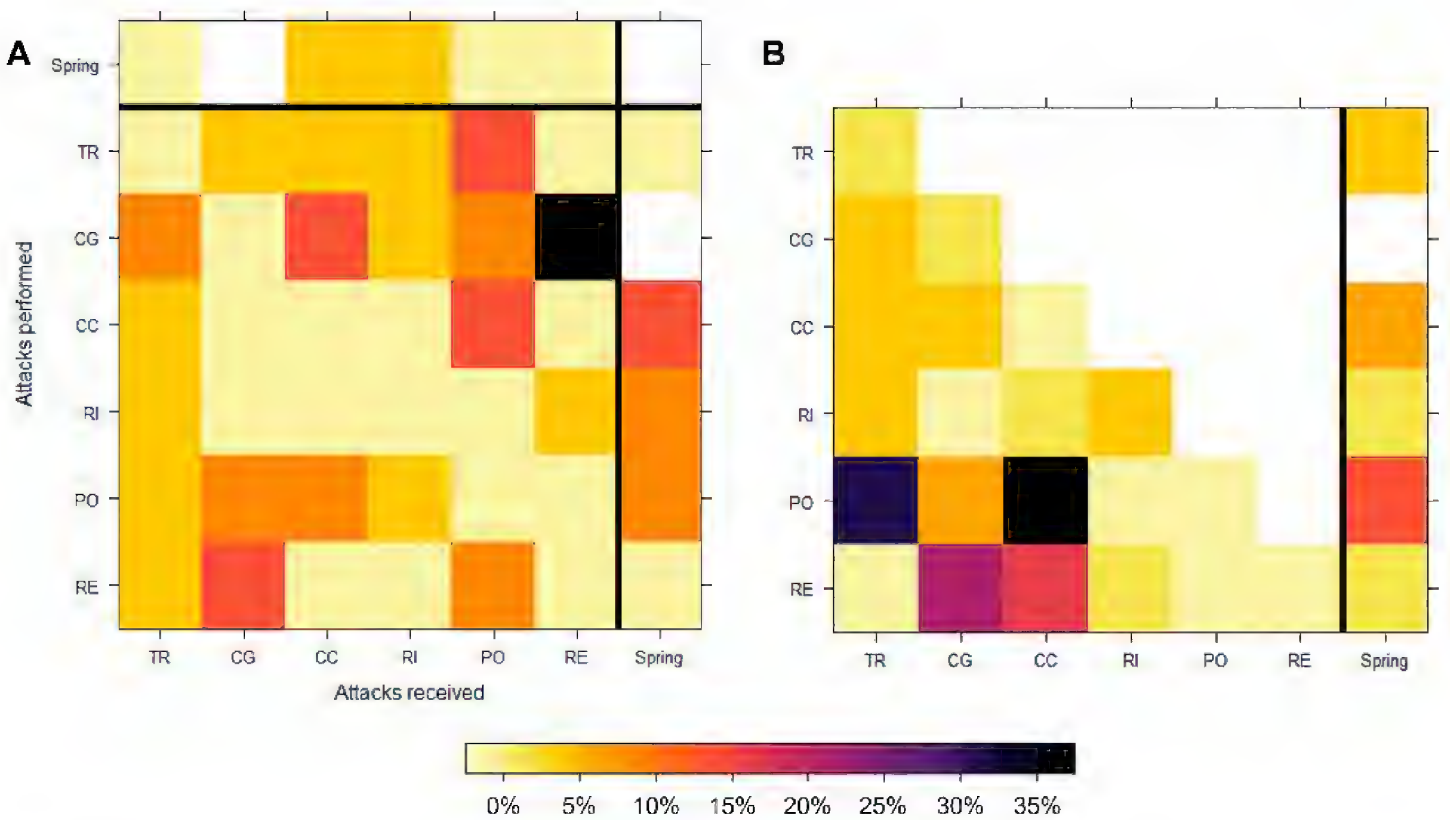

Figure 4. Proportion of attacks performed and received for the first time **A** and mortality **B** of *Linepithema humile* workers collected in Galiza in September towards workers from other nests collected in the same season, workers from the same nest and season (Control), and workers from the same nest but different season (spring). Note that when measuring mortality, nest ID could not be identified and therefore, corpses could belong to any of the two paired colonies. Workers from CG collected in March died before tests performed in autumn. Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

= 9.44, $p < 0.001$; Fig. 6). The mortality of *L. humile* workers confronted with *M. rubra* workers for 24h differed among nests in spring ($\chi^2 = 13.06$, df = 5, $p = 0.023$) but not in autumn ($\chi^2 = 4.29$, df = 5, $p = 0.509$). The mortality of *M. rubra* workers did not differ according to the *L. humile* nests they confronted in both seasons ($\chi^2 < 5.43$, $p > 0.365$).

According to the PCA results, nests showed no behavioural consistence across seasons (Suppl. material 1: Fig. S1) and thus, no particular nest behavioural pattern could be identified. For example, Reboreda nest showed the highest values of exploration and foraging (first forager and number of foragers) in spring, while the same nest in autumn showed low values for these behaviours. Ribeira and Pontevedra nests showed different behaviour in spring, while individuals from these same nests behaved similarly in autumn.

Genetic analyses

Allelic diversity in the *L. humile* genotyped populations ranged from 1 to 6 alleles per locus, with 38 alleles identified across all 7 loci. Significant deviations from Hardy-Weinberg equilibrium were found at all loci: *Lihu-S3* (Pontevedra); *Lhum-11* (Sant Cugat del Vallés, Pontevedra); *Lihu-T1* (Sant Cugat del Vallés, Cerdanyola del Vallés, Trabanca, Carril Garden, Ribeira, Pontevedra, Reboreda); *Lhum-13* (Sant Cugat del

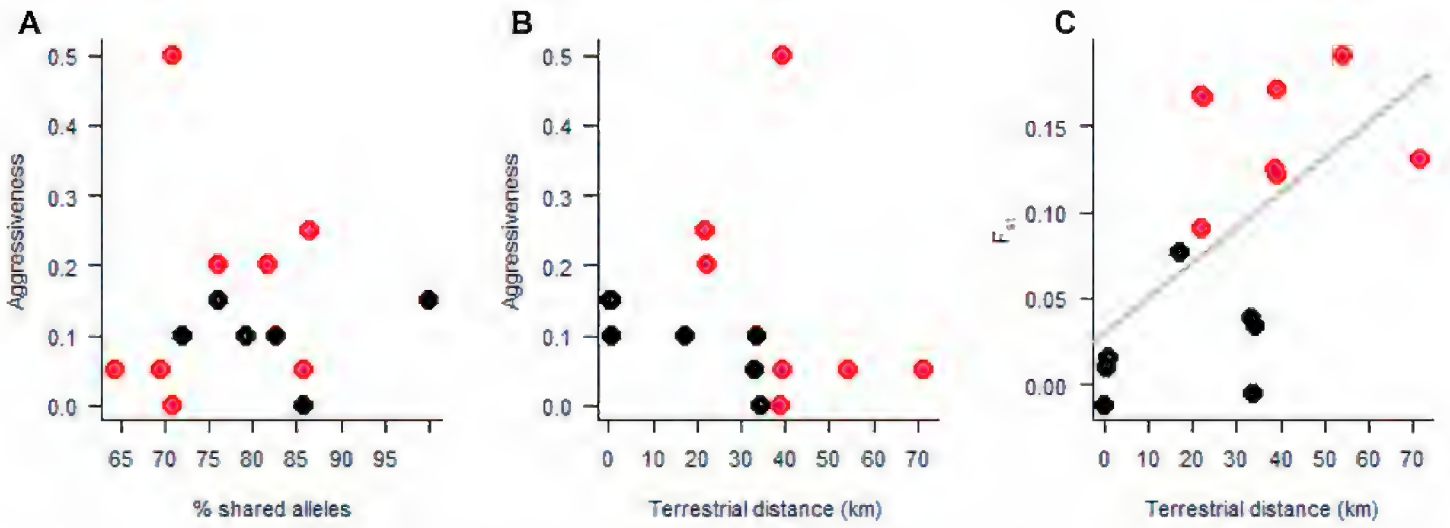

Figure 5. Relationships **A** between the percentage of shared alleles and the aggressiveness **B** between terrestrial distance and aggressiveness; and **C** between terrestrial distance and F_{ST}. Regression line is drawn for significant relationship (r = 0.57). Black dots represent pairs of colonies from the same genetic cluster, while red dots represent pairs of colonies from different genetic clusters. Note that these graphs include only populations sampled in Galiza.

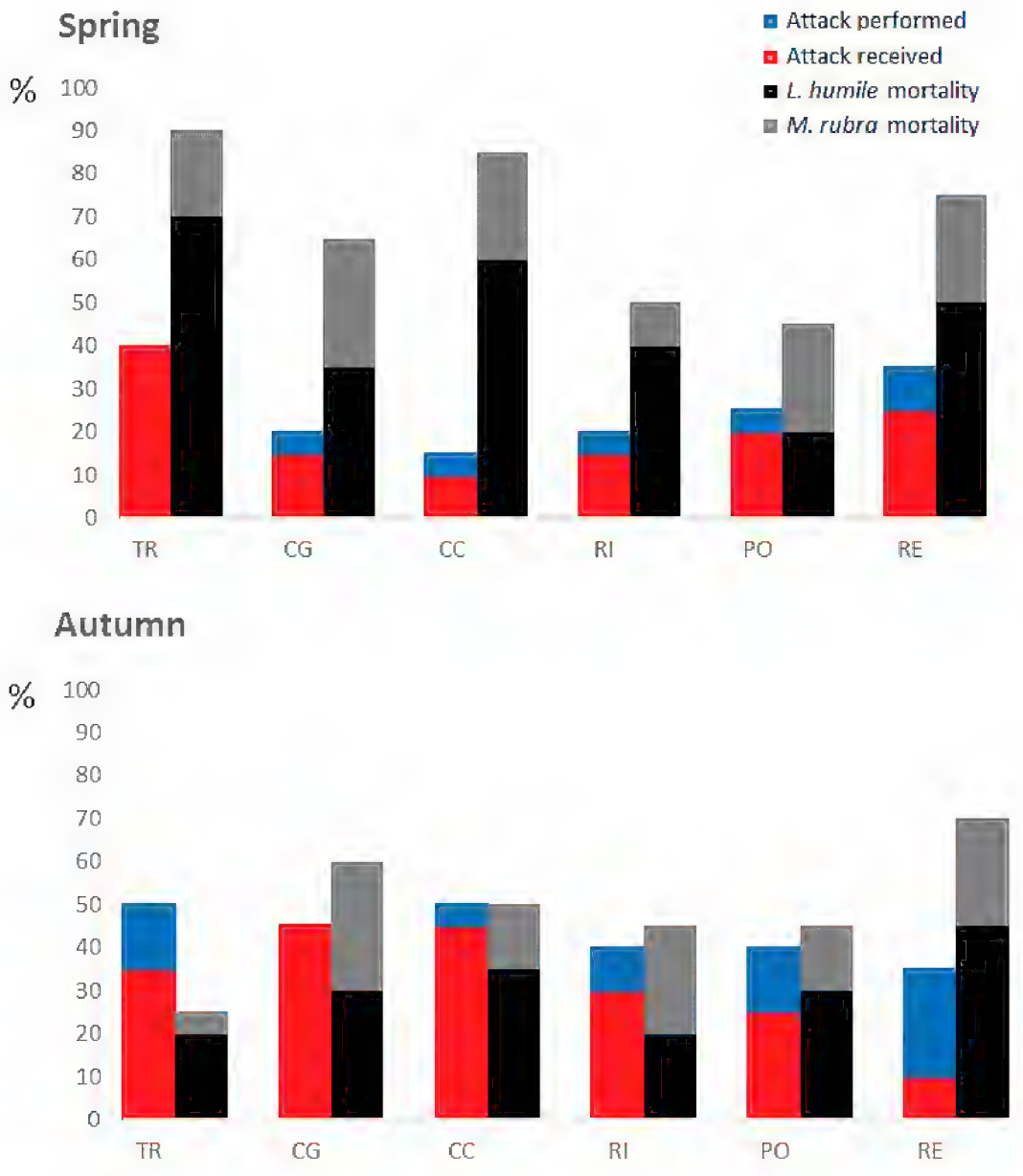

Figure 6. Proportion of attacks performed and received for the first time by *Linepithema humile* from Galiza and mortality after 24 hours paired for *L. humile* and *Myrmica rubra* in spring and autumn. Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

Table 3. Summary of genetic diversity for each of the *Linepithema humile* nests sampled for this study. For each locality, we list the mean number of alleles (Na), the mean observed heterozygosity (Ho), and total number of private alleles (Pa) across all seven microsatellite loci used for genotyping listed in Suppl. material 1: Table S1. Detailed information on genetic diversity indexes for each population/locus is provided in Suppl. material 1: Table S2.

Region	Locality	Supercolony	Na	Ho	Pa
Catalunya	Sant Cugat del Vallés	Catalonian	2.33	0.683	7
	Cerdanyola del Vallés	Main	3.5	0.714	2
Galiza	Trabanca	Main – North Cluster	3.2	0.656	2
	Carril Garden	Main – North Cluster	3	0.617	0
	Carril Coast	Main – North Cluster	3	0.562	0
	Ribeira	Main – North Cluster	2.9	0.623	0
	Pontevedra	Main – South Cluster	3	0.563	0
	Reboreda	Main – South Cluster	3	0.628	1

Vallés, Cerdanyola del Vallés, Trabanca, Carril Garden, Ribeira, Reboreda); *Lhum-19* (Trabanca, Carril Garden, Carril Coast); and *Lihu-M1* (Trabanca). A summary of microsatellite polymorphisms is presented in Suppl. material 1: Table S2. Within the main supercolony, the Catalonian locality (Cerdanyola del Vallés) showed slightly higher levels of observed heterozygosity over all loci and a higher number of alleles (Ho = 0.714 and 3.5 alleles over all loci, Table 3) than the localities sampled in Galiza (Ho mean±SD = 0.601±0.04 and a mean of 3.0±0.1 alleles over all loci). Levels of genetic diversity (i.e., observed heterozygosity and mean number of alleles) were similar between all the localities sampled in Galiza (Table 3).

Bayesian population assignment tests including all genotyped individuals (i.e., individuals from both main and Catalonian supercolonies sampled in Catalunya plus the individuals sampled in Galiza) identified K = 2 as the value that best fits the data. Results of the analyses with STRUCTURE assigned all individuals belonging to the Catalonian supercolony to one genetic cluster, well differentiated from the cluster that includes the *L. humile* individuals from Cerdanyola del Vallés (main supercolony) and all the localities sampled in Galiza (see Fig. 7, Suppl. material 1: Fig. S2). According to these results, all populations of *L. humile* sampled in Galiza would belong to the main supercolony. Nevertheless, at values of K = 3 (and even at K = 4; see Fig. 7), there is some level of genetic differentiation within the Galizan localities, with the populations of Pontevedra and Reboreda being clearly differentiated from the rest of the samples from the region (see Suppl. material 1: Figs S3, S4). In agreement with this, the STRUCTURE analyses including only the localities belonging to the main supercolony (i.e., excluding the samples from Sant Cugat del Vallés), identified two clusters of genetically similar individuals (see Suppl. material 1: Fig. S3): the first cluster includes the Cerdanyola del Vallés population in Catalonia, along with the localities of Trabanca, Carril (both Garden and Coast) and Ribeira in Galiza (hereafter the "North" cluster); and the second includes the populations of Pontevedra and Reboreda (hereafter the "South" cluster).

In agreement with the Bayesian clustering analyses results, the highest values of genetic differentiation (F_{ST}) were found between Sant Cugat del Vallés (Catalonian

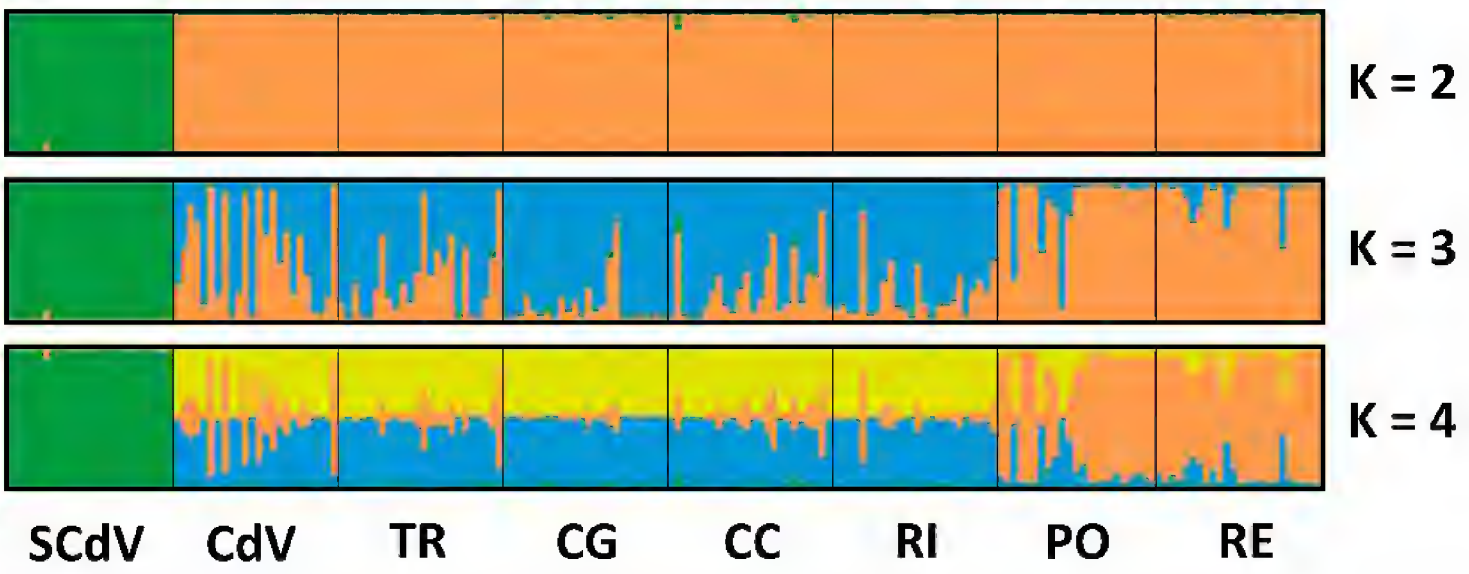

Figure 7. Genetic clustering of the eight *Linepithema humile* populations genotyped in this study, based on the seven microsatellite loci from Suppl. material 1: Table S1. Sampled localities are separated by black lines and each individual within the sampled localities is represented by a vertical bar. The proportion of colours in each bar indicates the genomic proportion derived from each genetic cluster. The plots presented here show that results of the STRUCTURE analysis with K = 2, K = 3 and K = 4. Populations from Galiza are listed according to the N-S geographical sampling gradient (see Fig. 1 and Table 1). Sant Cugat del Vallés: SCdV; Cerdanyola del Vallés: CdV; Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

supercolony) and the rest of the localities (i.e., main supercolony), yet pairwise F_{ST} values between the localities in the main supercolony were in most cases significant (except for the pairs Cerdanyola del Vallés – Carril Coast, Trabanca – Ribeira and Carril Coast – Carril Garden; see Table 4). These results indicate that a significant degree of differentiation exists among sampled localities, even within the same supercolony. Regarding the colonies from Galiza, F_{ST} values between each of the two genetic clusters identified by STRUCTURE (mean±SD; 0.13±0.06) were significantly higher than the F_{ST} values found within each cluster (0.02±0.03; t = 4.59, df = 10.84, $p < 0.001$; see Table 4). The levels of genetic similarity (i.e., the percent of shared alleles) were lower between the Catalonian and main supercolony (ranging from 16.7% alleles shared between Sant Cugat del Vallés and Ribeira to 25% alleles shared between Sant Cugat del Vallés and Reboreda), than between localities within the main supercolony. The percent of shared alleles within the latter was variable and ranged from 59.3% between Cerdanyola del Vallés and Redondela, to 100% between Carril Coast and Carril Garden (see Table 4). For the nests sampled in Galiza, the percent of alleles shared between nests belonging to the same genetic cluster (mean±SD; 83.03±9.02) was not significantly higher than the percent of alleles shared by nests belonging to different clusters (75.67±8.16; t = 1.65, df = 12.28, $p = 0.125$; see Table 4); but some differences in the distribution of allele frequencies could be observed between the North and the South cluster (see Suppl. material 1: Fig. S4).

For the localities sampled in Galiza, F_{ST} values were positively correlated with the geographical distance when considering either terrestrial distances (t = 2.52, df = 13, $p = 0.026$, r = 0.57; Fig. 5C), or the shortest distances between colonies (t = 3.97, df = 13, $p = 0.002$, r = 0.74).

Table 4. Population differentiation between the eight colonies of *Linepithema humile* included in this study, calculated with the data from the seven microsatellite loci listed in Suppl. material 1: Table S1. Values above diagonal represent percent of shared alleles between populations (i.e., the number of alleles shared across loci between localities/total number of alleles possessed by both localities). Values below diagonal correspond to F_{st} values. Values in bold indicate significant F_{st} values ($p < 0.05$). Negative values should be considered as zero. Sant Cugat del Vallés: SCdV; Cerdanyola del Vallés: CdV; Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

	SCdV	**CdV**	**TR**	**CG**	**CC**	**RI**	**PO**	**RE**
SCdV		19.4	22.6	24.1	24.1	16.7	16.1	25
CdV	**0.389**		63	63	65.4	79.2	75	59.3
TR	**0.345**	**0.048**		76	79.2	72	76	64.3
CG	**0.381**	**0.021**	**0.016**		100	86	81.8	71
CC	**0.398**	-0.029	**0.010**	-0.012		86	86.4	71
RI	**0.370**	**0.087**	-0.005	**0.039**	**0.034**		85.7	69.6
PO	**0.453**	**0.067**	**0.167**	**0.166**	**0.090**	**0.191**		82.6
RE	**0.383**	**0.114**	**0.121**	**0.171**	**0.125**	**0.131**	**0.077**	

Discussion

Our results support those from previous studies that identified the main supercolony in the NW of the Iberian Peninsula (Giraud et al. 2002), although the results of our population genetic analyses suggest the existence of genetic divergence in Galiza and identify two genetic clusters (i.e., North and South cluster; see Fig. 1 and Suppl. material 1: Fig. S3). The genetic differences and the aggressiveness found between these two clusters was lower than that expected between supercolonies, but higher than what would be expected within the same supercolony (Holway et al. 1998; Giraud et al. 2002; Suarez et al. 2002; Tsutsui et al. 2003; Thomas et al. 2005, 2006, 2007; Pedersen et al 2006; Vogel et al. 2009; Blight et al. 2010; Van Wilgenburg et al. 2010a; Blight et al. 2012, 2017, Berville et al. 2013) as it was found in *Myrmica rubra* (Chen et al. 2018) and *Formica pressilabris* (Hakala et al. 2020).

Aggressiveness tests performed with several populations of *L. humile* from Galiza suggested potential agonism within the main supercolony (X. Espadaler, personal communication 11 February 2021; pilot test performed by us, see Material and Methods). When considering all the genotyped nests, samples from Galiza are assigned to the same genetic group as the main supercolony (Fig. 7); however, when considering the Galizan samples and the main supercolony, i.e. excluding the Catalonian supercolony, our results suggest that the samples belonging to the main supercolony from Catalunya are more similar to the Galizan North cluster than to the South cluster (Suppl. material 1: Fig. S3). In agreement with this, F_{ST} values were also higher between than within both genetic clusters identified in Galiza. F_{ST} values found between Galizan clusters (0.13±0.06) were much lower than that expected between the main and the Catalonian supercolony (0.54; Jaquiéry et al. 2005; 0.47; Blight et al. 2012), lower than USA supercolonies (0.29±0.01; Thomas et al. 2006), close to the values found between native supercolonies (>0.15; Vogel et al. 2009; between 0.095±0.008 to 0.252±0.007;

Pedersen et al. 2006), and higher than those found between the main and Corsican supercolony (0.06; Blight et al. 2012). The between and within cluster F_{ST} values found in Galiza could fit within those found between (0.015 to 0.074) and within (0.034 to 0.103) supercolonies in *Formica pressilabris* (Hakala et al. 2020). However, the genetic diversity within each of the identified clusters was similar to that observed in the main supercolony, suggesting the maintenance of genetic diversity, albeit with differences in the distribution of alleles between clusters (see Suppl. material 1: Fig. S4).

Interspecific attacks were triggered by *L. humile* independently of the nest and season and these attacks were performed faster than the intraspecific attacks performed towards conspecifics from different nest. This suggest that ants were able to correctly identify their conspecifics but the inter-individual differences were sufficient to cause agonistic responses. We consider that the previous intraspecific aggressions observed in *L. humile* in the introduced areas (Tsutsui et al. 2000; Roulston et al. 2003) were due to the analysis of different supercolonies based on posterior studies that found other supercolonies in the studied areas (e.g. Buczkowski et al. 2004; Thomas et al. 2005). Therefore, we show for the first time the existence of aggressiveness within the same supercolony in *L. humile*. Although we did not find a clear pattern showing higher aggressiveness between the identified genetic clusters than within them, we found trends supporting this fact. For instance, up to 50% of attacks were registered between clusters and only up to 15% were registered within clusters. Furthermore, mortality was only found between clusters in spring while in autumn this was mainly found between clusters (up to 35% of cases) and less within them (<7.5%).

We found a significant correlation between genetical and geographical distances, with more distanced colonies being the most genetically different (Fig. 5C). This supports the definition of supercolonies proposed by Pedersen et al. (2006) in which they assumed a lack of individual flow between distanced nests of the same supercolony, and previous studies performed in California (Thomas et al. 2007). Higher relatedness between closer nests of the supercolony was proposed as sufficient to maintain kin-selection in *L. humile* although when local relatedness trends to zero (but see Vogel et al. 2009; Helanterä 2022). However, differences in aggressiveness could not be explained either by the geographical distances or the genetic dissimilarities between colonies (Fig. 5A, B). Similarly, no correlation between aggressiveness and geographical or genetic distances was found in other supercolonies (Giraud et al. 2002; Thomas et al. 2006, 2007; Vogel et al. 2009; but see Hakala et al. 2020).

All nests sampled in Galiza showed a higher expression of behaviors associated with invasiveness in autumn than in spring, except for the number of foragers, which was higher in spring. Seasonality determined workers' behavioural pattern: individuals were more proactive in autumn (more explorer, less thigmotactic, and more aggressive) than in spring. However, the number of foragers was higher in spring than in autumn. Foraging (forager abundance and recruitment) is highly dependent on the species, but also on temperature and habitat (Stuble et al. 2013). Our results could be interpreted according to differences in decision-making, which depends on the individual condition (Brodersen et al. 2008) and personality (Gambetti and Giusberti 2019). Proactive ants (emerged in

summer-autumn) facing resources could focus on carrying the resource because of their higher efficiency to do it, while reactive ants (emerged in winter-spring) due to their behavioural limitations, could focus on the group force or the recruitment of other workers.

As has been described in *Paratrechina flavipes* (Ichinose 1991), *L. humile* showed higher aggressiveness in autumn, when ants express higher activity levels. A similar trend was also found in *L. humile* introduced in the USA (Thomas et al. 2006, 2007). This suggests that workers' behaviour is conditioned by their biological cycle and/or the polydomy dynamics of the species. Lower temperatures diminish *L. humile* workers' activity and queens' fertility (Benois et al. 1973; Abril et al. 2018). Nests that remain separated in summer aggregate into hibernation nests, probably favoured by the activity slowdown in workers and queens (showed in California, Markin 1970; France; Benois et al. 1973; Argentina, Heller and Gordon 2006), the seasonal cuticular change (Abril et al. 2018), and the higher cost involved in defending a territory against multiple neighbour colonies (Giraud et al. 2002). On the other hand, the benefits in survivorship and fertility produced by aggregation (Luque et al. 2013) should also promote winter aggregations. In our experiment, ants fought similarly against *M. rubra* in both seasons, suggesting that seasonal differences in the intraspecific aggressiveness were not due to a reduction in the species agonistic behaviour but a mechanism mediated by the life history of the species. The seasonal effect could explain why we found aggressiveness in Galiza (only in autumn but not in spring) while previous studies performed over the same region carried out in spring (Giraud et al. 2002) did not find the same agonisms. However, aggressiveness tests were performed in other studies at different seasons and never found within supercolonies' agonism (native range: May (Blight et al. 2017), October-November (Vogel et al. 2009), December (Suarez et al. 1999); USA: April to September (Thomas et al. 2007), mainly in spring and summer (P. Nonacs, pers. comm. 2020), August, adding samples collected in the field every month for one year (Suarez et al. 2002), October (Thomas et al. 2006), November to March (Suarez et al. 1999); mainland Europe and islands: May (Blight et al. 2017), June (Wetterer and Wetterer 2006; Blight et al. 2010), October (Blight et al. 2012)). Future studies should decipher the underlying mechanism that drives the seasonal dependence of aggressiveness between colonies within the same supercolony.

We consider that our main results (low genetic differences, low aggressiveness within supercolony nests) support a better fit with the hypothesis of an evolutionary process of divergence in *Linepithema humile* linked to the development of agonistic interactions within the main supercolony rather than with the hypothesis of multiple introductions of native colonies. Aggressiveness within the same supercolony could be explained by differences in cuticular compounds caused by experienced local environmental factors as the diet (in *L. humile*, Liang and Silverman 2000; Buczkowski et al. 2005; but see Giraud et al. 2002), nest isolation (in *Lasius flavus*, Pontin 1961), season (in *Paratrechina flavipes*, Ichinose 1991) and/or genetic variability (Thomas et al. 2006; this study). Aggressiveness could act as a behavioural barrier limiting individual flow between nests and thus, increasing genetic differences between them. Evolutionary divergence was already suggested to explain the hypothetical emergence of the Corsican

supercolony from the main supercolony (Helanterä 2022), which begs the question as to whether the other European supercolony detected - the Catalonian supercolony - is also a product of an older divergence or an introduction of a second supercolony.

It must be taken into account that we have considered only two possible explanations for the existence of competition between *L. humile* nests located in the same region (new introductions and evolutionary divergence) due to the lack of within supercolony aggressiveness reported in previous studies and the rarity of new supercolonies founded by flying queens (Helanterä 2022). However, within supercolony competition could be produced without any of the cited mechanisms, and be more common than assumed (see Hakala et al. 2020). It was also shown that unicolonial species can be organized in aggressive supercolonies (Vogel et al. 2009); supercolonies could be constituted by a "fluid mosaic of aggressive and amicable interactions" (Hakala et al. 2020) between connected nests genetically and behaviourally differentiated (clusters or "subcolonies" according to Helanterä 2022). This could limit the functional unity of the introduced supercolonies (see Gordon 2010) and the connectivity of distanced nests (Pedersen et al. 2006). We could argue that the genetic and behavioural differences found could be also due to an ancient merging of supercolonies as assumed in *Lepisiota canescens* (Sorger and al. 2017) and *Formica paralugubris* (Holzer et al. 2009). However, this phenomenon seems unlikely due to the high aggressiveness and low individual flow between supercolonies of *L. humile*, as well as the relatively recent introduction of the species, which limits the time for colony merging. Another hypothesis not explored here is that the variability observed in Galiza is due to the introduction of a supercolony that was previously introduced in another region. Although we cannot discard this idea, we consider that the probability of introducing a different supercolony from the main supercolony is low due to the presence of this supercolony around the world (Van Wilgenburg et al. 2010b; Blight et al. 2012) and if this was the case, we would expect to find higher genetic variability than the one we found. Future studies should verify the possible origin of the genetic clusters found from other introduced supercolonies. In any case, we believe that our findings provide essential preliminary conditions for studies focusing on the future invasiveness of *L. humile* and those focused on the control of this species.

To conclude, our results point to divergent evolution as a possible cause of the incipient genetic divergence and behavioural variability found in the NW Iberian Peninsula. In addition, we showed a strong seasonal effect that conditions the expansion (exploration, use of open areas), efficiency (foraging), and aggressiveness of the nests of the sampled locations, suggesting competition within the supercolony. Considering the lack of competition within supercolonies as the main force of invasion for this species, our results showing agonism between nests of the same supercolony signal a weak point for this introduced species. In line with previous results, our study contributes to the development of conservation and management plans to control this species and to prevent the colonisation of new habitats. Conservation plans should be designed taking into account the season and the homogeneity of the nests, considering higher invasive potential for nests sharing similar traits and higher plasticity for those showing variability (Sanmartín-Villar

et al. 2021). Our findings could also contribute to a better understanding of the eco-ethology of the supercolony phenomenon and its evolutionary processes. Future studies should focus on understanding the origin of within supercolony variability.

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Supplementary material 1

Figures S1–S4, Tables S1, S2

Authors: Iago Sanmartín-Villar, Everton Cruz da Silva, Violette Chiara, Adolfo Cordero-Rivera, M. Olalla Lorenzo-Carballa

Data type: Docx file.

Explanation note: Genetic divergence and aggressiveness within a supercolony of the invasive ant *Linepithema humile*.

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Link: https://doi.org/10.3897/neobiota.77.90852.suppl1

Supplementary material 2

Behavioural data

Authors: Iago Sanmartín-Villar, Everton Cruz da Silva, Violette Chiara, Adolfo Cordero-Rivera, M. Olalla Lorenzo-Carballa

Data type: Data.

Explanation note: Data obtained from the behavioural tests.

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